Effects of follicle regulatory protein on thecal aromatase and 3 β -hydroxysteroid dehydrogenase activity in medium- and large-sized pig follicles

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Summary. Theca was excised from large (>8 mm) and medium-sized (3–6 mm) pig follicles and prepared as monolayer cultures in serum-free media. After 24 h cells were treated with (1) M199 (control), (2) 5 i.u. hCG, (3) 100 μ g or 100 ng FRP or (4) hCG (5 i.u.) + FRP (100 μ g or 100 ng). At 3, 6, 12, 24 and 48 h after treatment, progesterone, oestradiol, androstenedione and testosterone were measured in media. Formation of progesterone by microsomal fractions incubated (37°C) with 1 μ M-pregnenolone + 5 μ M-NAD⁺ for 1 h was used as a measure of 3 β -HSD activity. Aromatase activity was determined by incubating cells with [³H]testosterone for 3 h (37°C) and measuring ³H₂O release. In theca from large follicles, hCG enhanced 3 β -HSD activity after 48 h ($P < 0.05$) and secretion of progesterone after 36 h. FRP alone inhibited 3 β -HSD activity at 36 and 72 h, but had little effect on progesterone secretion. FRP inhibited ($P < 0.05$) the hCG-induced increase in 3 β -HSD activity at 36, 48 and 72 h. HCG enhanced aromatase activity after 48 h while FRP prevented ($P < 0.05$) the hCG-induced increase in aromatase activity at 48 and 72 h. Secretion of oestradiol was enhanced ($P < 0.05$) at 48 h but inhibited at 72 h by hCG. FRP alone had little effect on secretion of oestradiol but hCG + FRP was inhibitory at 72 h. Secretion of testosterone or androstenedione was unaffected by FRP. In theca from medium-sized follicles, hCG had little effect on 3 β -HSD at 27, 30, 36 and 48 h but was inhibitory at 72 h ($P < 0.05$). FRP alone inhibited 3 β -HSD activity at 72 h. Secretion of progesterone was stimulated by hCG after 36 h. FRP had little effect on levels of progesterone. HCG inhibited aromatase activity after 48 h. FRP had little effect on aromatase activity early after culture but was inhibitory at 48 and 72 h. Production of oestradiol was inhibited at 72 h by hCG while hCG + FRP were inhibitory at 48 and 72 h. Secretion of testosterone and androstenedione were unaffected by FRP in theca from medium-sized follicles. We conclude that FRP can inhibit hCG-induced 3 β -HSD and aromatase activity as well as steroidal secretion in thecal cells from large and medium-sized follicles of pigs.

Keywords: follicle regulatory protein; aromatase; 3 β -HSD; steroids; pig follicles

Introduction

The granulosa cell secretes a protein, referred to as follicle regulatory protein (FRP), which alters the response of developing follicles to stimulation by gonadotrophin (diZerega *et al.*, 1983). FRP has a molecular weight of ~ 15 000 and an isoelectric point of pH 4.5 (Holmberg *et al.*, 1986; Ono *et al.*, 1986). FRP has different biological properties from inhibin or folliculostatin since it does not inhibit release of FSH from rat pituicytes *in vitro* or *in vivo* (C. P. Channing, personal communications). This protein has been purified to homogeneity (Holmberg *et al.*, 1986; Ono *et al.*,

1986) and immunoassays have been established for its quantitation in biological fluids (Lew *et al.*, 1987).

Fractions of follicular fluid containing FRP have been shown to have a variety of effects on follicular function. In hypophysectomized immature rats treated with diethylstilboestrol, FRP inhibited the increase in ovarian weight and in peripheral concentrations of oestradiol in response to treatment with human menopausal gonadotrophin (diZerega *et al.*, 1982). When injected into regularly menstruating monkeys, FRP fractions decreased peripheral concentrations of oestradiol, caused prolongation of the follicular phase and anovulation or luteal phase dysfunction even in the presence of normal levels of gonadotrophins (diZerega & Wilks, 1984). Using pig granulosa cells, administration of fractions of follicular fluid inhibited induction of LH/hCG receptors by FSH (Montz *et al.*, 1984) as well as FSH-responsive adenylate cyclase activity (Ujita *et al.*, 1987). In addition, production and secretion of progesterone from rat and pig granulosa cells can be inhibited by FRP (Battin & diZerega, 1985a, b; Schreiber & diZerega, 1987). Using microsomal preparations from human placenta, and pig granulosa cells, FRP was shown to inhibit aromatase as well as 3β -hydroxysteroid dehydrogenase activities (Chicz *et al.*, 1985; Battin & diZerega, 1985a, b). Others have made similar observations, using fractions of follicular fluid, for pig (Guthrie *et al.*, 1986), cow (McNatty *et al.*, 1985), rat (Chari *et al.*, 1985) and mouse (Saito & Hiroi, 1986) follicles. Although FRP is produced by granulosa cells (diZerega *et al.*, 1983), it has been found in follicular fluid, serum and urine. Therefore, FRP can potentially affect other types of cells within the ovary.

Thecal steroidogenesis not only is important for providing substrate for production of steroids in granulosa cells but also for producing oestrogens, progestagens and androgens which in turn can affect follicular maturation. We have demonstrated stimulation of thecal aromatase and 3β -HSD activity by hCG (Tonetta *et al.*, 1986, 1987). Large quantities of oestradiol and progesterone inhibited enzyme activities while smaller quantities of oestradiol and progesterone stimulated enzyme activities in large follicles (Tonetta *et al.*, 1986, 1987; Tonetta, 1987). However, the effects of non-steroidal ovarian factors on thecal steroidogenesis are unknown. Therefore, we determined the effects of FRP on aromatase and 3β -HSD activity in thecal cells from large (>8 mm) and medium (3–6 mm)-sized pig follicles.

Materials and Methods

Materials. Human chorionic gonadotropin (hCG; Profasi HP10,000; lot 25325) was a gift from Serono Laboratories, Inc. (Randolph, MA). [$1,2\beta$ - ^3H]testosterone (sp. act. 48.3 Ci/mmol; lot 2139-055) was obtained from New England Nuclear (Boston, MA). The RIA kits for progesterone, oestradiol, androstenedione and testosterone were from Pantex (Santa Monica, CA) and Radio-Systems Laboratories (Carson, CA). The following reagents were purchased from Sigma Chemical Co. (St Louis, MO): collagenase (type II) from sheep testes; protease (type XIV) from *Streptomyces griseus*; epidermal cell growth factor from mouse submaxillary glands; insulin from bovine pancreas; oestradiol-17 β ; progesterone; pregnenolone; dihydrotestosterone (DHT). Medium 199 with Earle's salts, penicillin-streptomycin solution and amphotericin B were from Grand Island Biological Co. (Grand Island, NY). Human transferrin was from Pentex-Miles (Naperville, IL). All other reagents were obtained from commercial sources and were of the highest available grade of purity.

Follicular regulatory protein (FRP) was prepared from pig follicular fluid as previously described (Ono *et al.*, 1986).

Cell culture. Pig ovaries were obtained from the local abattoir. Follicles were determined to be healthy by gross appearance (translucent and vascularized). Thecal tissue was excised from large (diameter > 8 mm) and medium-sized (3–6 mm) follicles, placed in sterile, serum-free M199 and processed as previously described (Tonetta *et al.*, 1986). Briefly, cells were digested in 0.1% collagenase + 0.2% hyaluronidase for 30–40 min at room temperature. Cells were centrifuged (500 g, 28°C) for 30 sec and the supernatant, consisting mainly of granulosa cells, was discarded. The pellet was resuspended in 0.1% protease + 0.2% hyaluronidase in PBS-EDTA and incubated for 45 min at room temperature. The cells were washed 3 times in M199. After the last centrifugation, theca cells were resuspended in M199 + insulin (10 ng/ml) + transferrin (5 $\mu\text{g/ml}$) + epidermal growth factor 2.5 ng/ml). Viable cells were counted after trypan blue exclusion. Thecal cells were plated (5×10^5 cells/ml/well) in 24 multiwell plates and incubated at 37°C in 5% CO_2 , 95% air.

Fig. 1. Activity of (a) aromatase and (b) 3 β -HSD in thecal cells from medium and large-sized pig follicles. Values are mean $\pm$ s.e.m.

Treatment groups. At 24 h after plating, cells were treated as follows (total volume 1 ml): (1) control (M199); (2) hCG (Profasi, 5 i.u.); (3) FRP (100 ng or 100 μ g); (4) hCG (5 i.u.) + FRP (100 ng or 100 μ g). Media were removed at 3, 6, 12, 24 and 48 h after treatment and stored (-20°C) for determination of progesterone, oestradiol, testosterone and androstenedione by radioimmunoassay. The cells were used to measure thecal aromatase or 3 β -HSD activities. All cultures were repeated 3–4 times with 3–5 wells per treatment.

Aromatase activity. Aromatase activity was determined by a radiometric assay (Gore-Langton & Dorrington, 1981; Tonetta *et al.*, 1986). Since the conversion of [^3H]testosterone to oestradiol results in the release of $^3\text{H}_2\text{O}$, the percentage conversion of [^3H]testosterone to $^3\text{H}_2\text{O}$ reflects aromatase activity (expressed as percentage of controls). After removal of culture media, theca cells were incubated with 0.1 ml [^3H]testosterone (200 000 c.p.m.) plus 0.2 ml M199 for 3 h (37°C). Media were combined with 0.5 ml dextran-coated charcoal (0.5%) and incubated for 15 min. The tubes were then centrifuged (2000 g , 28°C) for 10 min, a 0.5 ml sample taken and placed in a vial with 9.5 ml scintillation fluid and counted. Interassay variability was $\sim 16.3\%$.

Activity of 3 β -hydroxysteroid dehydrogenase. Measurement of 3 β -HSD activity was performed by a modification of an assay previously reported (Chicz *et al.*, 1985) and independently validated (Tonetta *et al.*, 1987). After removal of culture media, 0.5 ml M199 was added to each well. Cells were scraped off the plates with a rubber policeman and wells were washed with another 0.5 ml M199. Cells were then sonicated and centrifuged at 10 000 g , (4°C) for 30 min. The supernatant was centrifuged (100 000 g for 60 min, 4°C) to obtain the microsomal pellet. M199 + 5 μM

Fig. 2. Secretion of (a) oestradiol, (b) progesterone, (c) testosterone and (d) androstenedione from thecal cells from medium- and large-sized pig follicles. Values are mean $\pm$ s.e.m.

NAD⁺ + 10⁻⁶ M-pregnenolone (total volume 1 ml) were added and the pellet was manually dispersed. The pellet was further sonicated to ensure even distribution of the microsomal material. Microsomes were incubated at 37°C for 1 h then heated at 60°C for 30 min. 3β-HSD activity was measured by conversion of pregnenolone to progesterone. Microsomal preparations from cells of representative wells were prepared in phosphate-buffered saline instead of M199 for protein determination by the method of Bradford (1976). Data are expressed as ng progesterone/h/μg protein. Interassay variability was ~15-4%.

Fig. 2 (continued).

Radioimmunoassays. Concentrations of progesterone, oestradiol, testosterone and androstenedione in media were determined by double-antibody radioimmunoassays. Generally, crossreactivity of the first antibody for other steroids was <0.1%. Samples were used without extraction. Media did not interfere with the measurements of steroids. The radioimmunoassays were validated by demonstrating parallelism in our assay systems (data not shown). Assay sensitivities were 2 pg/ml oestradiol, 39 pg testosterone/ml, 32 pg androstenedione/ml and 78 pg progesterone/ml.

Statistics. Statistical analysis was determined by one-way analysis of variance. Dunnet's test was used to compare treatments with controls while Bonferroni-T tests (Gill, 1978) were used to compare treatments.

Results

Although there was a dose-response using 100 ng and 100 µg FRP, overall, the results using 100 ng FRP were similar to those using 100 µg FRP. Therefore, only results using 100 ng FRP are presented.

Aromatase

Aromatase activity was unchanged in controls over time in theca from medium- and large-sized follicles. FRP enhanced ($P < 0.05$) aromatase activity at 72 h in theca from medium-sized follicles (Fig. 1a) while hCG inhibited aromatase activity after 48 h. FRP had little effect on the inhibition of aromatase activity by hCG in theca from medium-sized follicles. In large follicles, the addition of FRP had little effect on thecal aromatase activity while hCG enhanced ($P < 0.05$) aromatase activity after 48 h. However, FRP prevented the hCG-induced increase in aromatase activity in theca from large-sized follicles.

3 β -Hydroxysteroid dehydrogenase

Activity of 3 β -HSD increased from 36 to 72 h in theca from medium-sized follicles (Fig. 1b). HCG had little effect on 3 β -HSD activity but FRP inhibited ($P < 0.05$) 3 β -HSD activity at 72 h. FRP + hCG had similar effects to FRP alone on 3 β -HSD activity in theca from medium-sized follicles. In large-sized follicles, thecal 3 β -HSD activity increased from 27 to 48 h then decreased at 72 h. HCG enhanced ($P < 0.05$) 3 β -HSD activity in theca from large-sized follicles at 72 h, although the pattern of activity was similar to controls. FRP alone inhibited ($P < 0.05$) 3 β -HSD activity at 36 and 72 h. HCG + FRP decreased ($P < 0.05$) thecal 3 β -HSD activity at 36, 48 and 72 h compared to hCG-controls. In general, the 3 β -HSD system was more active in theca cells from large-sized follicles than from medium-sized follicles.

Oestradiol

Secretion of oestradiol by theca from medium-sized follicles was unchanged after treatment with FRP compared to controls (Fig. 2a). HCG had little effect on secretion of oestradiol from 27 to 48 h, but decreased ($P < 0.05$) concentrations at 72 h. The addition of hCG + FRP had effects similar to hCG alone. In theca from large-sized follicles, hCG enhanced ($P < 0.05$) secretion of oestradiol at 48 h but was inhibitory at 72 h. FRP alone had little effect on concentrations of oestradiol but hCG + FRP significantly decreased ($P < 0.05$) thecal production of oestradiol at 48 and 72 h compared to hCG alone.

Progesterone

In media from thecal cells of medium-sized follicles, concentrations of progesterone were relatively unchanged from 27 to 72 h (Fig. 2b). HCG, FRP and hCG + FRP had little effect. Thecal cells of large follicles increased concentrations of progesterone ($P < 0.05$) after 36 h in culture (Fig. 2b) and hCG enhanced ($P < 0.05$) secretion of progesterone at all times. FRP had little effect but prevented the hCG-induced increase in progesterone ($P < 0.05$) at 48 h.

Testosterone

Secretion of testosterone from thecal cells of medium-sized follicles increased from 27 to 36 h then decreased at 48 h (Fig. 2c). HCG had little effect on the secretion of testosterone at any time, and FRP, with or without hCG, had little effect. In general, medium-sized follicles secreted larger quantities of testosterone than did large-sized follicles. Concentrations of testosterone from theca of large-sized follicles decreased from 27 to 30 h then increased to a plateau. As in medium-sized follicles, hCG had little effect on secretion of testosterone. However, FRP decreased ($P < 0.05$) concentrations of testosterone at 30, 36 and 48 h of culture. The addition of hCG + FRP gave results similar to FRP or hCG alone.

Androstenedione

Concentrations of androstenedione in thecal media from medium-sized follicles were unchanged from 27 to 72 h (Fig. 2d). The addition of hCG, FRP or hCG + FRP had little effect on the secretion of androstenedione. Theca cells from large-sized follicles secreted more androstenedione than did cells from medium-sized follicles. Thecal production of androstenedione from large follicles increased ($P < 0.05$) from 27 to 36 h then decreased. HCG had little effect on secretion of androstenedione at all times tested while FRP decreased ($P < 0.05$) the secretion of androstenedione at 30 and 36 h. The addition of hCG + FRP decreased ($P < 0.05$) concentrations of androstenedione at 27 and 30 h compared to treatment with hCG alone.

Discussion

Theca cells from large-sized pig follicles have an active aromatase system which can be modulated by endogenous steroids (Tsang *et al.*, 1985; Tonetta *et al.*, 1986, 1987; Tonetta, 1987). As thecal cells may significantly contribute to peripheral concentrations of steroids including oestradiol (Tsang, 1984; Tsang *et al.*, 1985; Tonetta *et al.*, 1986) and thereby alter secretion of gonadotrophins, the regulation of thecal steroidogenesis may affect follicular maturation. FRP, which is secreted by granulosa cells (diZerega *et al.*, 1983), increased aromatase activity in thecal cells from medium-sized follicles after 48 h of treatment. However, in large follicles, FRP prevented the hCG-induced increase in aromatase which was reflected by a decrease in secretion of oestradiol. This suggests that the response of thecal cells to FRP differs as a function of follicular maturation.

These findings are in agreement with previous results which indicated that the effects of FRP on follicular cells are dependent upon the extent of follicular maturation and/or prior exposure to FSH. FRP increased basal secretion of progesterone in pig granulosa cells from small- and medium-sized follicles, but had no effect on granulosa cells from large follicles (Ledwitz-Rigby *et al.*, 1987). However, FRP inhibited the induction of progesterone by FSH in cultured rat and pig granulosa cells (Schreiber & diZerega, 1987; Ledwitz-Rigby *et al.*, 1987). The site of inhibition of progesterone by FSH is unclear in these cells as FRP inhibits the induction of adenylate cyclase by FSH in pig granulosa cells (Ujita *et al.*, 1987) and 3β -hydroxysteroid dehydrogenase activity in human placental microsomes (Chicz *et al.*, 1985).

3β -HSD activity in thecal cells from large-sized pig follicles is modulated by the endogenous steroids, oestradiol and progesterone (Tonetta *et al.*, 1987; Tonetta, 1987). As thecal progesterone provides substrate for thecal production of androgens as well as granulosa synthesis of oestradiol, regulation of thecal steroidogenesis by substances produced locally could influence follicular growth (Tonetta & diZerega, 1987). FRP alone had little effect on progesterone secretion from medium-sized follicles, but FRP inhibited 3β -HSD activity in thecal cells by 72 h of culture. However, in theca from large-sized follicles, FRP prevented the increase in thecal secretion of progesterone as well as 3β -HSD activity induced by hCG. FRP therefore appears to inhibit thecal steroidogenesis induced by gonadotrophin as the follicle matures. In granulosa cells, a biphasic response in granulosa and microsomal 3β -HSD activity was previously demonstrated by using preparations of pig follicular fluid (Ledwitz-Rigby *et al.*, 1987). Small doses inhibited while large doses enhanced induction of 3β -HSD activity by FSH. The increase in secretion of progesterone by pig granulosa cells treated with higher doses of FRP may be a result of an inhibition in 17-hydroxylase and 17-20 desmolase enzymes, thereby providing an increase in substrate rather than an apparent increase in 3β -HSD activity *per se*. However, FRP had little effect on the secretion of androgens from thecal cells.

The effect of granulosa FRP on thecal steroidogenesis is another example of cell-to-cell interaction within the follicle. Granulosa progesterone is a precursor for production of androgen by thecal cells of the rat (Lischinsky & Armstrong, 1983). In addition, charcoal-stripped media from cultures of pig thecal cells can inhibit granulosa steroidogenesis *in vitro* while media from

granulosa cultures can modulate thecal steroidogenesis (unpublished data). As FRP is secreted by granulosa cells from small- and medium-sized follicles (unpublished) and can modulate granulosa as well as thecal steroidogenesis, the action of FRP may be to restrict the maturation of follicular steroidogenesis in adjacent follicles, thus inducing or allowing for generalized atresia. Although secretion of FRP does not appear to be regulated by gonadotrophins or steroids (unpublished), the action of FRP on the cell is altered by the presence of FSH. In pig granulosa cells *in vitro*, there is a shift in responsiveness to FRP depending upon the dosage of FSH present; larger quantities of FSH can overcome the inhibitory effects of FRP on granulosa steroidogenesis (Schreiber & diZerega, 1987). Therefore, follicles destined to ovulate may overcome the inhibitory effects of FRP due to their prior exposure to or intrafollicular concentrations of FSH.

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